Nasally delivered chitosan-coated poly(lactide-co-glycolide) encapsulating honeybee venom enhances T helper 1-related immunity against *Salmonella* Typhimurium infection in pigs

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OBJECTIVE

*Salmonella* Typhimurium is a significant zoonotic concern for human food poisoning and a substantial economic burden in the swine industry. We previously reported that nasally delivered chitosan-coated poly(lactide-co-glycolide) (PLGA) encapsulating honeybee venom (CP-HBV) could enhance CD4+ T helper 1 (Th1)-related immune responses in healthy pigs. Building upon these findings, the current study aimed to investigate the protective immune enhancement by nasally delivered CP-HBV in pigs challenged with *S* Typhimurium.

ANIMALS

36 healthy, 4-week-old, female, Landrace X Yorkshire X Duroc pigs.

METHODS

36 pigs were allocated into 3 groups: CP-HBV (n = 16), control (n = 16), and healthy baseline control (n = 4). CP-HBV and control groups were challenged with *S* Typhimurium 7 days post-treatment. Pigs from the healthy control group were sacrificed at 0 days postinfection (DPI), and 4 pigs from each of the control and CP-HBV groups were sacrificed at 1, 2, 4, and 7 DPI. *Salmonella* shedding, immune cell frequencies, cytokines, and transcriptional factor expression levels were measured.

RESULTS

The CP-HBV group exhibited lower bacterial shedding and an enhanced Th1-related immune response characterized by an upregulation of CD4+ T cells and CD4+ Interferon-γ+ T cells, accompanied by increased expression of Th1-related cytokines and reduced expression of regulatory T cells and immunosuppressive cytokines compared to the control group.

CLINICAL RELEVANCE

CP-HBV is a promising strategy for controlling *Salmonella* infections in pigs and improving public health.

Keywords: honeybee venom, immune enhancement, nanoparticle, protective immunity, *Salmonella* Typhimurium

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variety of parenteral drugs, proteins, and vaccines. These nanoparticles allow for the controlled release of encapsulated antigens, which can stimulate immune cells over an extended period. However, the use of PLGA in nasal drug delivery systems has been limited due to its lack of mucoadhesive properties. To overcome this limitation, we formulated nanoparticles, PLGA coated with chitosan, a natural polysaccharide comprising copolymers of glucosamine and N-acetylglucosamine, which has been shown to enhance mucoadhesion and prolong antigen release profiles at mucosal sites to make it an ideal complement to PLGA for nasal drug delivery applications. Several studies have demonstrated the enhanced induction of immune responses by mucoadhesively modified PLGA particles with chitosan on mucosal surfaces and in the systemic circulation.

Preparation of CP-HBV

HBV and CP-HBV used in the current study were aseptically prepared the same as previously described. Briefly, PLGA (200 mg) was dissolved in 5 mL of dichloromethane at room temperature. Separately, HBV (15 mg) was dissolved in 0.5 mL of distilled water at room temperature. The 2 solutions were emulsified using a homogenizer for 5 minutes at 20,000 RPM, followed by probe sonication for 2 minutes in an ice bath at an amplitude of 20% to obtain a water-in-oil emulsion. The resulting water-in-oil emulsion was then injected into 20 mL of a solution containing 0.5% (w/v) polyvinyl alcohol (molecular weight [MW], 13,000 to 23,000) and 0.7% (w/v) low-molecular-weight chitosan (50.190 kDa, 75% deacetylation) in acetate buffer (pH, 4.4). The mixture was subjected to sonication for 6 minutes at an amplitude of 35% to form a water-in-oil-in-water emulsion. The dichloromethane was subsequently evaporated by stirring the emulsion at 100 RPM overnight at room temperature. The PLGA nanoparticles were isolated by centrifugation at 17,000 X g for 20 minutes at 4 °C and washed once with distilled water. The resulting CP-HBV was lyophilized and stored at 4 °C until further use. As a control, PLGA nanoparticles without chitosan coating were prepared using the same procedure. All materials used in preparation of CP-HBV were obtained from Sigma-Aldrich.

Animals

Conventional 4-week-old pigs (Landrace X Yorkshire X Duroc; Daehan Livestock & Feed) were sourced from a single healthy herd with no prior history of S Typhimurium infection. All animals were housed in air-conditioned facilities and provided ad libitum access to a nutritionally complete, antibiotic-free diet and drinking water. Prior to the study, all pigs were screened and confirmed negative for Salmonella by bacteriological culture of fecal samples. The entire study was conducted in accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal experimental procedures were reviewed and approved by the IACUC of Chonnam National University (Approval number: CNU IACUC-YB-2013-29).

Experimental design

Pigs (weight range, 4 to 8 kg) were randomly allocated into 3 experimental groups based on balancing body weight between each group: 1) CP-HBV group (n = 16; average body weight, 6.36 kg), which received 21 mg/10 mL of CP-HBV intranasally; 2) control group (n = 16; average body weight, 6.07 kg), which received an equivalent volume of sterile PBS intranasally; and 3) healthy control group (n = 4; average body weight, 6.47 kg), which remained untreated and served as a baseline. Due to the complex anatomy of the pigs’ intranasal area, it is possible that a small amount of the formulation (likely less than 0.5 mL) may have been lost during administration. Seven days after CP-HBV administration, pigs from both the control and CP-HBV groups were orally challenged with 10 mL of S Typhimurium (American Type Culture Collection) at a concentration of 1 X 10⁹ CFU/mL. The healthy control group received sterile medium. Prior to the experiment at 0 days postinfection (DPI), 4 pigs from the healthy control group were sacrificed to determine baseline immune status. At 1, 2, 4, and 7 DPI, 4 randomly selected pigs from both the control and CP-HBV groups were sacrificed by IV administration of a high dose of pentobarbital (40 mg/kg), followed by exsanguination. During necropsy, fecal samples and tissue samples from the ileum, cecum, colon, MLNs, and spleen were collected for Salmonella counts and further immunological analyses. Peripheral whole blood was collected in EDTA-coated tubes for CBC (IDEXX Laboratories) analysis. A schematic representation of the experimental design is depicted (Figure 1).
Viable bacterial count in fecal and tissue samples

All samples were homogenized (10% w/v) in PBS, and 10-fold serial dilutions were prepared. Each dilution was plated in triplicate onto xylose lysine deoxycholate agar (BD Biosciences) supplemented with 100 μg/mL kanamycin (BD Biosciences) and incubated at 37 °C for 48 hours. Characteristic black colonies were enumerated for the dilutions, yielding 30 to 300 colonies per plate and expressed as CFU per gram of feces or tissue.

Isolation of mononuclear cells

To isolate mononuclear cells (MNCs) from the ileum and MLNs, tissues were minced and digested in RPMI-1640 medium (Lonza) supplemented with 2 mg/mL collagenase/dispase (Roche Diagnostics) and 5% fetal bovine serum (FBS) (Lonza) at 37 °C with periodic agitation for 40 minutes. The cell suspension was sequentially passed through 100-μm and 40-μm cell strainers (BD Biosciences) and centrifuged at 500 X g for 10 minutes. The cell pellet was resuspended in RPMI-1640 medium (Lonza) containing 5% FBS (Lonza) and layered on an OptiPrep (Stemcell Technologies) density gradient. Following centrifugation at 750 X g for 30 minutes with the brake disengaged, the light density fraction was collected and washed twice with ice-cold PBS. The washed cells were resuspended in RPMI-1640 medium (Lonza) containing 5% FBS (Lonza) and 2% antibiotics-antimycotics (Lonza). Cell viability and counts were determined by trypan blue dye exclusion using a hemocytometer.

Phenotypic identification of MNCs

Flow cytometry analysis was performed to define different immune subsets as previously described. MNCs (5 X 10⁶) were washed with ice-cold PBS and incubated for 30 minutes with the following antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-pig CD3ε (clone BB23-8E6-8C8; BD Biosciences), R-phycoerythrin (PE)-cyanine 7 (PE/Cy7)-conjugated anti-pig CD4a (clone 74-12-4; BD Biosciences), and PE-conjugated anti-pig CD8a (clone 76-2-11; BD Biosciences) To identify regulatory T cells (Tregs), cells (5 X 10⁶) were first stained with FITC-conjugated anti-pig CD4a (clone 74-12-4; BD Biosciences) and anti-porcine CD25 (clone K231.3B2, Bio-Rad) at 4 °C for 30 minutes, followed by PE/Cy7-conjugated anti-mouse immunoglobulin G1 (clone M1-14D12; eBioscience). Cells were then fixed and permeabilized using an intracellular fixation and permeabilization buffer (eBioscience) and subsequently incubated with PE-conjugated anti-mouse/rat Foxp3 (clone FJK-16s; eBioscience) for 30 minutes. Stained cells were acquired and analyzed using an Accuri C6 flow cytometer (BD Biosciences).

Measurement of interferon-γ–producing CD4⁺ T cells

Approximately 1 X 10⁶ MNCs were stimulated with a cell stimulation cocktail (containing phorbol 12-myristate 13-acetate and ionomycin; eBioscience) for a total of 5 hours, with a protein transport inhibitor cocktail (containing brefeldin A and monensin; eBioscience) added during the final 2 hours of incubation. Following stimulation, cells were harvested and stained with FITC-conjugated anti-pig CD3ε (clone BB23-8E6-8C8; BD Biosciences), PE/Cy7-conjugated anti-pig CD4a (clone 74-12-4; BD Biosciences), and PE-conjugated anti-pig CD8a (clone 76-2-11; BD Biosciences). Cells were then fixed and permeabilized using an intracellular fixation and permeabilization buffer (eBioscience) and subsequently incubated with Alexa Fluor 647-conjugated anti-pig interferon-γ (IFN-γ) (clone P2G10; BD Biosciences) for 30 minutes. Stained cells were acquired and analyzed using an Accuri C6 flow cytometer (BD Biosciences).
Quantitative real-time PCR

Total RNA was extracted from tissue samples using Trizol (Invitrogen) and the PureLink RNA Mini Kit (Invitrogen) according to the manufacturers’ instructions. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo-Fisher Scientific), and RNA purity was assessed by determining the absorbance ratio at 260 and 280 nm. RNA integrity was further confirmed by visualizing the 18S and 28S ribosomal RNA bands following electrophoresis on 0.7% agarose gels (Lonza). Equal amounts of total RNA were reverse transcribed using the LeGene cDNA Synthesis Master Mix (LeGene). To minimize variations in reverse transcription efficiency, all samples were transcribed simultaneously. Quantitative PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on a MyiQ2 thermocycler with the SYBR Green detection system (Bio-Rad). The quantitative PCR conditions were as follows: initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of denaturation at 95 °C for 30 seconds, annealing at 57 °C for 30 seconds, and extension at 72 °C for 30 seconds. Each sample was run in duplicate. Primer sequences are listed (Table 1). The threshold cycle (Ct), defined as the cycle number at which the amplified target gene amount reaches a fixed threshold, was determined for each reaction. The relative quantitation value for each target gene was normalized to the endogenous control gene (β-actin) and expressed as 2-ΔΔCt (fold change), where ΔCt = Ct of the target gene − Ct of the endogenous control gene, and ΔΔCt = ΔCt of samples for the target gene − ΔCt of the calibrator for the target gene.

Statistical analysis

Statistical analyses were performed using Prism, version 10 (GraphPad Software). Data are expressed as mean ± SD. Comparisons of different parameters between the control and CP-HBV groups were performed using an unpaired t test. Parameters measured throughout the infection period were compared to those of the healthy control group using a one-way ANOVA followed by the Dunnett multiple comparison test. P values < .05 were considered statistically significant.

Results

Bacterial clearance of S Typhimurium in challenged pigs

Feces and tissue samples from the ileum, cecum, colon, MLNs, and spleen, which are known to be the primary target site for Salmonella infection, were collected for S Typhimurium isolation at 0 DPI from the healthy control group and 1, 2, 4, and 7 DPI from control and CP-HBV treated group. Salmonella counts peaked at 1 DPI in all isolated sites, including feces, ileum, cecum, and colon, except for MLNs and spleen, which peaked at 2 and 4 DPI, respectively. Following the peak, Salmonella counts continuously decreased until the end of the experiment (Figure 2). The CP-HBV treated group effectively controlled the bacterial burden, exhibiting significantly lower Salmonella counts in feces and intestinal organs throughout the entire experimental period compared to the control group (P < .05 or P < .01).

Changes in systemic immune cells in S Typhimurium–challenged pigs

Peripheral whole blood was collected in EDTA-coated tubes at 0 DPI from the healthy control group and 1, 2, 4, and 7 DPI from the control and CP-HBV groups for further analysis. The absolute immune cell counts in peripheral blood were monitored by CBC after the infection (Figure 3). In the control group, all immune cell populations decreased after infection, except for eosinophils and basophils. The reduction was particularly pronounced in lymphocytes and

Table 1—The quantitative PCR primer sequences.

<table>
<thead>
<tr>
<th>Sequence (5’–3’)</th>
<th>Accession number</th>
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<td>CCCGCCGAAGATATAACTGA</td>
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<td>IL-10 FW</td>
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<td>TGF-β FW</td>
<td>CAGGCTCTGAGGAGCAT</td>
</tr>
<tr>
<td>β-actin FW</td>
<td>CAGGATCGATAGAAGAAGT</td>
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</tbody>
</table>

FW = Forward primer. IFN-γ = Interferon-γ. IL-1β = Interleukin-1β. RV = Reverse primer. STAT4 = Signal transducer and activator of transcription 4. T-bet = T-box transcription factor TBX21. TGF-β = Transforming growth factor-β. TNF-α = Tumor necrosis factor-α.

*GenBank accession number and corresponding gene are available online at http://www.ncbi.nlm.nih.gov/.
Figure 2—Salmonella counts after S Typhimurium challenge in pigs. S Typhimurium colonies were isolated from feces, ileum, cecum, colon, mesenteric lymph nodes (MLNs), and spleen, enumerated, and normalized to grams of tissue or feces. Statistical analyses were performed to compare the control group versus the encapsulating honeybee venom (CP-HBV) group and the healthy control (baseline) group versus the control group. *P* values are presented as indicated; otherwise, differences were nonsignificant.

Figure 3—Changes in peripheral blood immune cells after S Typhimurium challenge in pigs. Peripheral blood samples were collected at 0, 1, 2, 4, and 7 days postinfection and analyzed by CBC to monitor changes in the absolute numbers of peripheral immune cells. Statistical analyses were performed to compare the control group versus the encapsulating honeybee venom (CP-HBV) group and the healthy control (baseline) group versus the control group. *P* values are presented as indicated; otherwise, differences were nonsignificant.
monocytes, with these populations remaining suppressed in the control group throughout the entire experimental period ($P < .01$ or $P < .001$). In contrast, the CP-HBV treated group exhibited significantly higher lymphocyte and monocyte counts, especially at 2 ($P = .030$ and $P = .031$, respectively), 4 ($P = .021$, only for lymphocytes), and 7 DPI ($P = .039$, only for lymphocytes) compared to the control group.

**Early immune cell dynamics in the ileum and MLNs of S Typhimurium–challenged pigs**

We sought to further understand how S Typhimurium manipulates local immune responses and whether CP-HBV could alter the immunomodulatory actions induced by S Typhimurium. On the day of necropsy, ileum and MLN tissues were collected from each pig, and MNCs were isolated using a density gradient method for further immune cell phenotyping and measurement of IFN-γ producing CD4+ T cells. First, we explored Th1-related immune cell response in the ileum and MLNs (Figure 4), where the first site serves as immune surveillance against S Typhimurium infection.5,21 The frequency of CD4+ T cells in the ileum from the control group decreased at 1 DPI compared to baseline ($P = .006$); however, this reduction was quickly recovered, although the frequency remained lower than the baseline. The MLNs exhibited similar trends in CD4+ T-cell frequency as the ileum, but the changes did not reach statistical significance. In contrast, the CP-HBV treatment group demonstrated a significantly higher level of CD4+ T cells in both the ileum and MLNs, especially at 2 ($P = .027$ and $P = .040$, respectively), 4 ($P = .032$ and $P = .034$, respectively), and 7 DPI ($P = .010$, only for ileum) compared to the control group. Furthermore, IFN-γ secreting CD4+ T cells gradually increased in the ileum from both the control and CP-HBV groups after infection; however, the CP-HBV group maintained a significantly higher frequency than the control group at 2 ($P = .016$), 4 ($P = .036$), and 7 ($P = .037$) DPI. In the MLNs, no substantial changes were observed in IFN-γ secreting CD4+ T cells in either the control or CP-HBV group compared to baseline, although the CP-HBV treatment group showed a higher frequency at 4 DPI compared to the control group ($P = .014$).

Next, we explored the changes in the frequency of Tregs to determine if they were altered by S Typhimurium infection (Figure 4). We observed a

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**Figure 4**—Changes in immune cell frequencies in the ileum and mesenteric lymph nodes (MLNs) after S Typhimurium challenge in pigs. Ileum (A) and MLN (B) tissues were harvested on the day of necropsy, and cells were isolated for further immunological analysis. Isolated cells were stained and analyzed by flow cytometry. CD4+ T cells were defined as CD3+CD4+CD8−, and regulatory T cells (Tregs) were defined as CD4+CD25+Foxp3+. To measure interferon-γ (IFN-γ) secreting CD4+ T cells, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin, and then the expression level of IFN-γ in CD4+ T cells was measured by flow cytometry. Statistical analyses were performed to compare the control group versus the encapsulating honeybee venom (CP-HBV) group and the healthy control (baseline) group versus the control group. $P$ values are presented as indicated; otherwise, differences were nonsignificant. Foxp3 = Forkhead box P3.
substantial increase in Treg frequency in the ileum from the control group at 1 DPI, which was nearly 4-fold higher ($P < .0001$) compared to the baseline. Interestingly, the frequency of Tregs in the MLNs did not increase at 1 DPI in the control group compared to the baseline; instead, it exhibited a significant increase at 2 DPI ($P < .0001$). In contrast, the CP-HBV treatment group demonstrated a significantly lower expression of Tregs in the ileum compared to the control group at 1 ($P = .045$) and 2 DPI ($P = .043$) and in the MLNs at 2 ($P = .010$), 4 ($P = .017$), and 7 DPI ($P = .016$).

**Cytokine and transcriptional factor expression in the ileum and MLNs of *S Typhimurium*-challenged pigs**

At the day of necropsy, ileum and MLN tissues were harvested from each pig, and total RNA was extracted to measure the levels of cytokines and transcription factors in the ileum and MLNs of *S Typhimurium*-challenged pigs. Total RNA was extracted from the ileum (A) and mesenteric lymph nodes (B), and real-time PCR was performed to measure the mRNA expression levels of proinflammatory (tumor necrosis factor-α [TNF-α] and Interleukin-1β [IL-1β]), T helper 1-related (Interferon-γ [IFN-γ], IL-12, T-box transcription factor TBX21 [T-bet], signal transducer and activator of transcription 4 [STAT4]), and regulatory T-cell related (IL-10 and Transforming growth factor-β [TGF-β]) cytokines and transcription factors. Values were normalized to the healthy control group and presented as fold changes. Statistical analyses were performed to compare the control group versus the encapsulating honeybee venom (CP-HBV) group and the healthy control (baseline) group versus the control group. $P$ values are presented as indicated; otherwise, differences were nonsignificant.

**Figure 5**—Changes in mRNA expression of cytokines and transcription factors in the ileum and MLNs after *S Typhimurium* challenge in pigs. Total RNA was extracted from the ileum (A) and mesenteric lymph nodes (B), and real-time PCR was performed to measure the mRNA expression levels of proinflammatory (tumor necrosis factor-α [TNF-α] and Interleukin-1β [IL-1β]), T helper 1-related (Interferon-γ [IFN-γ], IL-12, T-box transcription factor TBX21 [T-bet], signal transducer and activator of transcription 4 [STAT4]), and regulatory T-cell related (IL-10 and Transforming growth factor-β [TGF-β]) cytokines and transcription factors. Values were normalized to the healthy control group and presented as fold changes. Statistical analyses were performed to compare the control group versus the encapsulating honeybee venom (CP-HBV) group and the healthy control (baseline) group versus the control group. $P$ values are presented as indicated; otherwise, differences were nonsignificant.

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transcription factors using real-time PCR. We first assessed the mRNA expression of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) and Interleukin-1β (IL-1β) (Figure 5). In the ileum, the levels of TNF-α and IL-1β increased in both the control and CP-HBV groups, with the CP-HBV group exhibiting significantly higher expression compared to the control group at 1 (P = .017 and P = .001, respectively), 2 (P = .013 and P = .021, respectively), and 4 DPI (P = .025, only for TNF-α). The CP-HBV treated group also showed a significant increase in TNF-α and IL-1β in MLNs compared to the control group at 1 (P = .011 and P = .024, respectively), 4 (P = .045 and P = .021, respectively), and 7 DPI (P = .010 and P = .027, respectively).

Next, we investigated the expression levels of Th1-related cytokines and transcription factors, such as IL-12, IFN-γ, T-box transcription factor TBX21 (T-bet), and signal transducer and activator of transcription 4 (STAT4) (Figure 5). Both the control and CP-HBV groups exhibited a gradual increase in Th1-related cytokines and transcription factors in the ileum, with the highest peak for cytokines at 4 DPI and for transcription factors at 2 DPI. The CP-HBV group demonstrated increased expression of all measured cytokines and transcription factors throughout the entire experimental period, particularly at 2 DPI, with an approximately 2-fold higher expression compared to the control group (P = .001 for IFN-γ, P = .0007 for IL-2, P = .008 for T-bet, and P = .005 for STAT4). The expression levels of the Th1-related cytokines and transcriptional factors in MLNs were comparatively lower than in the ileum, even in the CP-HBV group. The control group maintained a lower expression than the baseline throughout the entire study period. In contrast, the CP-HBV group exhibited a higher level of expression compared to the control group, particularly at 1 DPI (P = .001 for IFN-γ, P = .002 for IL-2, P = .014 for T-bet, and P = .021 for STAT4).

Given the limited evidence regarding the role of Tregs in S Typhimurium infection in pigs, we further investigated the mRNA expression of Treg-related cytokines, including IL-10 and Transforming growth factor-β (TGF-β), which serves as immunosuppressive cytokines (Figure 5). The control group showed a significantly higher expression of IL-10 and TGF-β in the ileum at 1 (P < .0001 and P < .0001, respectively), 2 (P < .001 and P < .0001, respectively), and 4 DPI (P = .001 and P = .0005, respectively) and in the MLNs at 4 DPI (P = .006 and P = .001, respectively) compared to the baseline. The CP-HBV group demonstrated decreased expression of IL-10 and TGF-β in the ileum compared to the control group at 1 (P = .039 and P = .045, respectively), 2 (P = .016 and P = .032, respectively), and 4 DPI (only for IL-10, P = .040) and in the MLNs at 4 (only for TGF-β, P = .025) and 7 DPI (P = .034 and P = .028, respectively), suggesting that CP-HBV treatment might attenuate the immunosuppressive response induced by S Typhimurium infection.

**Discussion**

Here, we demonstrated that nasally delivered CP-HBV enhanced proinflammatory and Th1-related immune responses and weakened Treg-related immune-suppressive action in S Typhimurium-challenged pigs, which might lead to rapid bacterial clearance. The early immune response to *Salmonella* in local tissues, such as the ileum and MLNs, involves the recruitment of neutrophils and inflammatory monocytes to produce proinflammatory cytokines, including TNF-α and IL-1β, which are essential in delaying the spread of bacteria to systemic tissues. In concert with recruited neutrophils and inflammatory monocytes, macrophages within infected tissues phagocytose *Salmonella* and recognize cytosolic flagellin, inducing the production of IL-1β and IL-18. Indeed, we found a massive increase in proinflammatory cytokines, such as TNF-α and IL-1β, in the ileum and MLNs upon *Salmonella* infection; meanwhile, its secretion was strengthened by CP-HBV treatment.

Interestingly, *S Typhimurium* was not detected in the spleen at 1 DPI, unlike other collected tissues, likely because *S Typhimurium* initially invaded local intestinal lymphoid tissues before disseminating systematically to peripheral blood or distant organs via efferent lymphatic vessels. Moreover, both the control and CP-HBV groups exhibited a slight decrease in the number of lymphocytes and monocytes in peripheral blood at 1 DPI, suggesting that a large proportion of these cells might have recruited to the local target site upon *Salmonella* infection as *Salmonella*-specific T-cell activation typically commences in the ileum within 3 hours postinfection and in the MLNs within 9 to 12 hours.

Several studies have highlighted the importance of Th1-related immune responses in bacterial clearance and prevention of bacterial persistence following *S Typhimurium* infection, driven by IFN-γ, IL-12, and TNF-α. We observed a notable increase in CD4+ T cells and IFN-γ secreting CD4+ T cells following CP-HBV treatment, concurrent with high expression of Th1-related cytokines and transcription factors, such as IFN-γ, IL-12, T-bet, and STAT4, indicating that Th1-related immune enhancement by CP-HBV could contribute to rapid bacterial clearance. Another interesting finding in the present study is the upregulation of immunosuppressive cytokines alongside an increased frequency of Tregs after *Salmonella* infection in pigs. Tregs were initially defined as a CD25-expressing subset of CD4+ T cells secreting CD4+ T cells following CP-HBV treatment, concurrent with high expression of Th1-related cytokines and transcription factors, such as IFN-γ, IL-12, T-bet, and STAT4, indicating that Th1-related immune enhancement by CP-HBV could contribute to rapid bacterial clearance.

In conclusion, we confirmed that pigs treated with nasally delivered CP-HBV exhibited accelerated *S Typhimurium* clearance in the early stage...
of infection, possibly due to an augmented Th1-specific immune response and reduced Treg-related immunosuppression. The early immune response to *Salmonella* in the ileum and MLNs involves the recruitment of neutrophils and inflammatory monocytes, producing proinflammatory cytokines like TNF-α and IL-18, which are crucial for delaying bacterial spread to systemic tissues. Our findings confirmed that CP-HBV treatment further enhanced the secretion of TNF-α and IL-18. Additionally, CP-HBV led to an increase in CD4+ T cells, IFN-γ secreting CD4+ T cells, and high expression of Th1-related cytokines and transcription factors, contributing to rapid bacterial clearance. Interestingly, *Salmonella* infection also induced an upregulation of immunosuppressive cytokines and increased the frequency of Tregs, which was reduced in the CP-HBV treated group. These findings suggest that CP-HBV could be a promising alternative or complementary strategy for controlling *Salmonella* infections in pigs.

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**Disclosures**

The authors have nothing to disclose. No AI-assisted technologies were used in the generation of this manuscript.

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